Development of refractoriness of HO-1 induction to a second treatment with UVA radiation and the involvement of Nrf2 in human skin fibroblasts

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ABSTRACT

UVA treatment of cultured human skin fibroblasts (FEK4) has been shown previously to reduce transcriptional activation of hemeoxygenase 1 (HO-1) following a second dose of UVA radiation, a phenomenon known as refractoriness. This study demonstrates that the levels of HO-1 protein are also reduced after a second dose of UVA radiation as are Nrf2 levels, and there is less accumulation of Nrf2 in the nucleus whereas Bach1 does accumulate in the nucleus. Cell viability is further reduced and cell membrane damage increased as compared with a single UVA treatment when an initial UVA treatment was followed by a second dose. Knockdown of Nrf2 by siRNA (siNrf2) targeting caused additional refractoriness of HO-1 protein induction to a second UVA or heme treatment and this treatment also further enhanced cell damage by a second dose of UVA radiation. However, transfection with Nrf2 caused less refractoriness of HO-1 to a second dose of UVA and reduced cell damage by a second dose of UVA radiation. These findings are consistent with the proposal that Nrf2 is involved in HO-1 refractoriness and could serve as a cytoprotective factor against cell damage caused by repeated exposure to moderate doses of UVA radiation. We propose that protection by the Nrf2-HO-1 pathway protection may have clinical relevance since human skin is exposed repeatedly to UVA radiation.

INTRODUCTION

Ultraviolet A (UVA) radiation (320-400 nm) is a major part of solar ultraviolet light (>90%) and causes an oxidative stress which has deleterious effects on human skin, lead to erythema, photoaging and even cancer (1,2). Among antioxidant defence mechanisms identified in skin is the enzymatic antioxidant, heme oxygenase 1 (HO-1, Enzyme Classification Number: 1.14.99.3) (3), which catalyses heme to yield carbon monoxide, biliverdin and iron (1,4). HO-1 is a sensitive marker of oxidative stress, and is induced by UVA-irradiation of human skin fibroblasts (3) and many other cell types (1,5). Induction of HO-1 gene expression involves transactivation by a bZIP transcription factor Nrf2 (nuclear factor erythroid-2 p45-related factor 2) which forms a heterodimer with Maf (small Maf family of proteins) and binds to the antioxidant response elements (AREs) in the HO-1 gene promoter region. Conversely, Bach1 (BTB and CNC homology-1), an additional bZIP factor, forms a Bach1-Maf complex and functions as a repressor of HO-1 gene transcription (1,6,7). Our previous studies have shown that UVA induces HO-1 gene expression, but does not alter HO-2 gene and protein expression in human skin fibroblast (FEK4) cells (3,5). Treatment of chicken embryo liver cells with heme leads to development of refractoriness to HO-1 gene activation by a second heme treatment (8). Refractoriness of HO-1 gene induction to a second dose of UVA radiation was also observed in FEK4 cells (9). However, the refractoriness of HO-1 has not been examined at the protein level and the mechanism of development of refractoriness is yet to be fully defined (8,9).

Nrf2 has been shown to play a pivotal role in preventing xenobiotic-related toxicity and oxidative stress. The protective role of Nrf2 involves the induction of Phase II detoxification enzymes as well as antioxidant enzymes, such as HO-1 through the ARE (6,7). Nrf2 drive- HO-1 expression has been shown to be protective in human lukemia as well as skin diseases (10,11). We have recently found that Nrf2 plays an active role in both UVA radiation and heme induced HO-1 induction, and loss of Nrf2 sensitises FEK4 cells to UVA-radiation induced membrane damage as reflected by

63 enhanced LDH release (12,13). Bach1 plays a negative role in HO-1 induction in both
64 skin keratinocytes and fibroblasts (13-15). Since induction of HO-1 following UVA
65 irradiation is modulated by Nrf2 and Bach1 proteins, both therefore contribute to the
66 balance of cellular redox status (1,15).

67 In this study, we investigate if there is a refractoriness to HO-1 protein induction
68 corresponding to the refractoriness to HO-1 mRNA accumulation. In addition, we
69 investigated the role of Nrf2, an upstream transcriptional activator of the HO-1 gene, in
70 refractoriness and undertook a preliminary study of the role of Bach1 in the
71 refractoriness of HO-1. Our results are consistent with a link between Nrf2 and HO-1
72 refractoriness and demonstrate that deficiency of Nrf2 protein further increases cell
73 damage by a second dose of UVA irradiation. We also have some evidence that Bach1
74 is also likely to be involved in HO-1 refractoriness. These results provide a better
75 understanding of the pathophysiological effects of UVA irradiation on human skin cells,
76 and once similar studies are available *in vivo*, will help to evaluate the effects of
77 repeated physiological exposures of the skin to UVA radiation as well as repeated
78 clinical UVA phototherapy.
79

MATERIALS AND METHODS

Cell Culture and Antibodies: Human primary skin fibroblasts (FEK4) cells were grown in Earle's modified Minimal Essential Medium (MEM). The MEM medium was enriched with 15% (v/v) FCS and 2 mM*L*-glutamine along with 50 units/mL of penicillin and streptomycin mixture. Antibodies against HO-1 (OSA-110) and HO-2 (OSA-200) were purchased from the Bioquote International (UK).Three antibodies (Anti-Nrf2 H300, sc-13032; anti-Bach1 C-20, sc-14700; anti-actin sc-9104) and three secondary antibodies (anti-goat, -rabbit and -mouse IgG) made by Santa Cruz Biotechnology (USA) and Sigma-Aldrich Chemical Co. (UK), respectively, were used (12-15).

Irradiation of Cells with UVA: Sellas (Germany) made broad spectrum (4-kW) lamp was used to irradiate cells following standard procedures (12,13) while non-irradiated cells were used as a background control (sham = 0 kJ/m^2). After incubation of UVA-irradiated cells for a period of time (48-72 h), the cells were re-irradiated, and then incubated in a saved conditional medium (cMedium) for the required time. The basic protocol for the re-dosing regimen is set out in Table 1.

Table 1.The basic protocol for the re-dosing regimen interval time (24-72h)

1st dose(1^{o})	**2nddose (2^{o})**	**Symbol**
Sham	Sham	Sham(-/-)
Sham	UVA	-/UVA (-/+)
UVA	Sham	UVA/- (+/-)
UVA	UVA	UVA/UVA (+/+)

<Table 1>

RNA Interference by siNrf2: Sequences of all small interference RNAs (siRNAs) against Nrf2 are as follow:

No.1 s9491 Sense: 5'-GAAUGGUCCUAAAACACCAtt-3'

Antisense: 5'-UGGUGUUUUAGGACCAUUCtg-3'

No.2 s9493 Sense: 5'-CAGUCUUCAUUGCUACUAAtt-3'

Antisense: 5'-UUAGUAGCAAUGAAGACUGgg-3'

Cells were transfected with two different concentrations of siNrf2 (5 and 30 nM) and scrambled control (Sb, 30 nM) at the time of plating as follow: After trypsinization, cells in suspension were transfected with scrambled oligonucleotides control (Sb, AM4611) (Ambion) and siNrf2 using the siPORT™ NeoFX™ Transfection Agent (AM4511, Ambion) using the different steps. The siRNA dilution were made in 100 µl OPT-MEM and 5 µl NeoFX in 100 µl OPT medium respectively, uniformly mixed and incubated together at room temperature (RT) for 10 min until the siRNA complexes are formed. The siRNA complex was taken in 6- cm plates and a medium containing $3x10^5$ cells were added to make a final volume of 2.5 ml. After about 24 hours, the plates were additionally given 1 ml of 15% (v/v) of FCS-MEM fresh medium and again incubated for 48-72 h prior to further treatment (12-15).

Transient transfection: Cells were seeded into 96-well and 6- cm plates (in order to perform RT-PCR of HO-1 and confirm transfection of Nrf2) 40 h before transfection to reach 60% confluency. They were then transfected with pcDNA3.1-Nrf2 and its control vector using the transfection reagent Lipofectamine 2000 (Invitrogen), in a 1:2 volume ratio (DNA:Lipofectamine) at RT according to the manufacturer's instructions. The DNA–Lipofectamine complex was incubated with cells in Optimum (OPT) medium (Invitrogen) for 6 h and then 1:1 growth medium was added for 6 h, finally this medium was replaced with growth medium for a further 34 h (total 40 h) before UVA irradiation (12, 15).

RNA isolation, reverse transcription, and quantitative real-time PCR: Total RNA was collected from sham- and UVA-irradiated cultured skin cells using an RNA extraction kit, following the supplier's instructions. RNA samples were quantified and reverse transcription was performed (15). Quantitative real-time PCR primers were as follows: HO-1, forward (F): AAGAGGCCAAGACTGCGTTC; reverse (R), GGTGTCATGGGTCAGCAGC; Nrf2, F: GCGACGGAAAGAGTATGAGC R:

GTTGGCAGATCCACTGGTTT; GAPDH, F: GACATCAAGAAGGTGGTGAA; R, TGTCATACCAGGAAATGAAG. RT-PCR was carried out with a Roche LightCycler 1.5 instrument using the SYBR green assay (Roche). A standard curve was created using serial dilutions of a pooled sample of cDNA. Gene expression levels are presented as arbitrary units normalized to the expression of the housekeeping gene GAPDH (15).

Treatment of Cells with Heme: Cells in the presence of hemin (ferri-protoporphyrin IX) were incubated in cMedium for 1 h to generate heme (ferrous-protoporphyrin IX). Cells were rinsed with PBS two times, and then cMedium was added and the cells incubated for the next 48 h. Cells were re-treated again with hemin for 1 h, washed twice using PBS and re-incubated with cMedium for the prescribed intervals.

To reduce the variations due to cell division, the freshly confluent FEK4 cells (95-100%) were employed as described previously (9): Cells were treated with UVA radiation, heme (1 h) or cadmium (1 h), re-incubated in cMedium for the indicated times (interval times) and then were exposed to a second treatment of these agents.

Protein extraction and western blotting: Standard methods (12-14] were used to extract proteins from the harvested cells following treatment. Equal amounts of total protein lysate (30-50 µg depending on the experiment) and loading buffer were mixed to separate on a 10% SDS-PAGE. A second gel was run using the identical protein lysate as a loading control. After separated proteins were shifted onto PVDF (Millipore) membranes and probed with actin (1:3000), Nrf2 (1:200), Bach1 (1:400) and HO-1 (1:200) antibodies, following a standard protocol (12-14). Subsequently, chemiluminescence makes the protein bands visible on X-ray film by using the ECL Western blotting detection system (Invitrogen). Digital densitometry was done to quantify the intensity of protein bands by using the program NIH Image J1.33. Actin was used to compare and normalize the data with respective controls and presented as the fold change adjusted to 1.

Immunocytochemistry: Cells were grown to sub- or just freshly confluent on glass cover-slips, collected and rinsed with PBS following fixation in 4% (w/v)

paraformaldehyde then incubated in 100% methanol at -20ºC. The Image-iT™ Fx signal enhancer (Invitrogen) was used to block the cells and then were treated with 1:100 Nrf2 and 1:200 Bach1 antibody and then Alexa-Fluor secondary antibody. Hoechst nuclear stain was applied and the cover-slips mounted. For analysing the cells, oil immersion epi-fluorescence Nikon Eclipse TE2000-U microscope was used and finally the images were taken using UltraVIEW program.

LDH measurement: The levels of extracellular lactate dehydrogenase (LDH) were monitored by using the ready to use cytotoxicity detection kit for LDH (Cat. No. 11644793001, Roche Applied Science) according to supplier instructions. Cells after treatment with SiNrf2 (7500) were seeded into 96-well plates for 48 h, then irradiated with UVA, incubate for 4 h and finally LDH release was measured according to Zhong et al. (12). LDH release was calculated as the extracellular LDH as a fraction of total LDH. Data was represented as the fold increase in LDH release over sham-irradiated, scrambled siRNA or vector- treated control.

MTS assay: After treatment as described in the LDH assay, 20 µl of MTS reagent (The Cell Titer 96®AQ_{ueous} Non-Radioactive Cell Proliferation Assay reagent Promega Cat. No. G5421) were added to monitor the absorbance at 490 nm after the optimal time (1.5 h post incubation). The absorbance peaks, are the measure of the cell viability, were used to compare sham-irradiated cells with that of scrambled siRNA or vector- treated control, were set to 100.

Statistical analysis: Two-tailed T-test was applied to analyse the data and a *P*-value <0.05 was taken to be statistically significant value. The descriptive statistics [mean, standard errors (SE)] is presented graphically.

RESULTS

HO-1 protein is induced by UVA irradiation

HO-1 is an oxidative stress-inducible protein whose expression is highly inducible in human primary skin fibroblast FEK4 and other cell lines, when treated with a large number of physical stressors (e.g., UVA irradiation) and chemical (e.g., H_2O_2, hemin) agents (1,3,16). HO-1 protein levels following the various doses of UVA irradiation in FEK4cells has not examined before. To confirm the dose response for the inducibility of HO-1 in FEK4 cells, we examined the protein levels of HO-1 by western blotting at 8 h following UVA irradiation in the range of 50 to 500 kJ/m^2. This study also included longer incubation times (3 to 72 h) than a previous study (9). As expected, the induction of HO-1 protein by UVA is dose-dependent. A maximal induction of HO-1 protein was observed in FEK4 cells that were exposed to 250 kJ/m^2 UVA radiation. However, the protein level declined with exposure to a higher dose of radiation (500 kJ/m^2) (Fig. 1A). HO-1 protein induction post a moderate dose, i.e., 250 kJ/m^2 has not examined before, so a time course of HO-1 protein induction was determined during 72 h following treatment with a moderate dose (250 kJ/m^2) of UVA radiation. As shown in Fig. 1B, the maximal increase of HO-1 protein (12±1.2 folds) was observed 12 h post irradiation and HO-1 level remains higher than basal until at least 48 h and then decreased to basal levels by 72 h.

<Figure 1>

Refractoriness of HO-1 protein to re-induction occurs with UVA and heme but not cadmium

A Western blotting assay showed that HO-1 protein levels returned to the basal levels 60-72 h after single UVA treatment (Fig. 1). Total protein was collected between 8-12 h following the second treatment since during this period UVA radiation shows 8-12 fold HO-1 induction (Fig. 1B) (9,12,14). The levels of HO-1 protein were significantly reduced in the pre-irradiated cells as compared with the non pre-irradiated samples when the interval time was up to 48 h (Fig. 2A, middle panel, P <0.05). Indeed, an interval time of 72 h was required to obtain maximal reduction in HO-1 protein levels,

i.e. from 12 fold (-/UVA) to 6 fold (UVA/UVA) induction of HO-1 (Fig. 2A, right panel, $P < 0.05$). No reduction of HO-1 induction was observed when the interval time is 24 h (Fig. 2A, left panel, $P > 0.05$) and after an interval time of 96 h the HO-1 refractoriness is strongly reduced as compared with 72 h (data not shown). A second treatment with heme induced a modest, yet significant reduction of HO-1 protein levels as compared with cells that have not been pre-treated with heme, i.e., 9 fold vs. 6 fold ($P < 0.05$, Fig. 2B). However, with a second cadmium (Cd) treatment or a cadmium pre-treatment following by UVA radiation, HO-1 levels were not reduced (Fig. 2C). A positive control demonstrated that the refractoriness response to UVA was normal i.e., 10 fold (-/UVA) vs. 6 fold (UVA/UVA) at this 60 h interval time (Fig. 2B and C, $P < 0.05$). After these treatments, HO-2 remains at a constant level (Fig. 2A and data not shown).

<Figure 2>

Refractoriness of Nrf2 activation to a second UVA treatment develops after an initial UVA exposure

Nrf2 has been implicated in the induction of HO-1 protein levels in skin fibroblasts by both UVA radiation and heme (12). We hypothesise that the refractoriness to re-induction of HO-1 protein by UVA might be associated with the altered Nrf2 and Bach1 accumulation in the nucleus following a second dose of UVA radiation. We thus examined sub-cellular localisation and total protein levels of both Nrf2 and Bach1 following a second dose of UVA radiation. In order to perform immunostaining for the localisation of Nrf2 and Bach1, a 48 h (but not 72 h) interval time was chosen between two irradiations to avoid cells being too confluent and to facilitate cell morphology studies. At this time (48 h), refractoriness to HO-1 mRNA accumulation was maximal (9) but maximal refractoriness to HO-1 protein induction occurred a few hours later (Fig. 2A). Both Nrf2 and Bach1 translocations were monitored at 2, 4 and 8 h following a second dose of UVA irradiation and compared to levels observed after a single dose of UVA radiation (Fig. 3). Fig. 3A showed that Nrf2 did not accumulate in the nucleus

at 2, 4 and 8 h following a second dose of UVA irradiation and it stays mostly in the cytosol, whereas nuclear accumulation of Nrf2 did occur in cells that had not been irradiated previously. Increased nuclear accumulation of Bach1 was observed following a second spell of UVA irradiation (Fig. 3B).

Furthermore, Nrf2 protein levels in whole cell lysates were significantly reduced following the second dose of UVA irradiation, i.e. 3 fold (UVA/UVA) vs. 5 fold (-/UVA) Nrf2 induction 8 h following UVA (Fig. 3D); whereas Bach1 protein levels were significantly higher, i.e. 3.3 fold (UVA/UVA) vs. 2 fold (-/UVA) (Fig. 3E). The reduced Nrf2 and increased Bach1 levels corresponded temporarily with reduced HO-1 levels (Fig. 3F). Reduced Nrf2 levels were also observed at the 72 h interval time (data not shown) and these were comparable to those seen with the 48 h interval time. The nuclear and cytosolic fraction were used and found that the Nrf2 levels are increase even more in nuclear when compared with total cellular level (data not shown),but the total cellular levels of Nrf2 reflect the nuclear increase of Nrf2, therefore was used for the following experiments (12).

<Figure 3>

Nrf2 may involve in the refractoriness of HO-1 protein to induction by a second treatment with UVA irradiation and heme

In a previous study, it has been shown that Nrf2 was involved in up-regulating HO-1 following UVA radiation and heme treatment and silencing of Nrf2 led to a reduction in both UVA- and heme induced HO-1 levels (12,13). We studied whether modulation of Nrf2 would effect a second treatment with either UVA radiation or heme with respect to the refractoriness to induction of HO-1 protein in human dermal fibroblast FEK4 cells.

First silencing of Nrf2 was performed and Nrf2 protein knockdown by siRNA was confirmed. Concentrations of 5 and 30 nM siNrf2 caused a reduction of up to 80% of original protein levels (12). The level of HO-1 that is induced in cells, which had been given siNrf2 were further reduced, i.e. from 7 fold of the scrambled control to 5

fold (5 nM siNrf2) and 2.5 fold (30 nMsiNrf2) 12 h following the second dose of UVA radiation (Fig. 4A, P <0.05). Similarly, siNrf2 treatment has significantly decreased the level of HO-1 induction by a second dose of heme (Fig. 4B, P <0.05). A second siNrf2 reagent that targeted a different exon in Nrf2 (Ambion, No. 2) showed similar results (data not shown).

Nrf2 overexpression was confirmed and we observed that the concentrations of 0.5 and 2 μg Nrf2 caused an increase of up to 10-fold of basal mRNA levels (data not shown). The level of induction of *HO-1* mRNA at 6 h following UVA irradiation (with 48 h interval time between irradiations) was shown to be 16- fold compared to the sham control. The expression of HO-1 in cells, which had been transfected with Nrf2 was increased, i.e. from 8- fold of the vector control to 11- fold (0.5 μg Nrf2) and 15- fold (2 μg Nrf2) 6 h following the second dose of UVA radiation (Fig. 4C, P <0.05).

<Figure 4>

Nrf2 may play a role in protection against a second treatment with UVA radiation

Nrf2 is implicated in protection of many cell types against oxidative damage since it is involved in up-regulating detoxifying phase-II enzymes, such as HO-1. A previous study indicated that cells treated with UVA radiation twice, have the same extent of free iron release as the first dose and thus may cause the same extent, if not more cell membrane damage in FEK4 cells (17). We next examined whether loss of Nrf2 further increased damage induced by a second UVA treatment, using LDH leakage and cell viability to measure the damage. Our results show that a second UVA treatment (UVA/UVA) significantly increased membrane damage to 2.6- fold (Fig. 5A, P <0.05) and decreased cell viability to 79% (Fig. 5B, P < 0.05) when compared to the situation in cells that had received a single dose of UVA (-/UVA). Furthermore, a second UVA treatment caused both LDH leakage increase and cell viability loss and this damage was further exacerbated by Nrf2 knockdown i.e., LDH leakage increased from 2.6- fold (column 4) to 3- fold (column 6) and 3.6- fold (column 7) with 5 nM and 30 nM siNrf2 pre-treatment, respectively (Fig. 5A, P < 0.05). Also, cell viability decreased from 79%

(column 4) to 73% (column 6) and 65% (column 7) with 5 and 30 nM siNrf2 pre-treatment, respectively (Fig. 5B, P <0.05). Both types of cell damage increase in a siNrf2 concentration dependent manner.

We then tested whether an increase in Nrf2 levels reduced damage following a second UVA treatment, using LDH leakage and cell viability to measure the damage. Our results showed that the level of both increased LDH leakage (Fig. 5C) and cell viability loss caused by a second UVA treatment (Fig. 5D) were reduced by Nrf2 overexpression i.e., LDH leakage reduction from 2.5- fold (vector control: column 3) to 2.3- fold (column 4) and 1.9- fold (column 5) with 0.5 and 2 μg Nrf2 transfection, respectively (Fig. 5C, $P < 0.05$), while a single UVA treatment caused a ~2- fold LDH release when compared with sham control as observed previously (12). We observed cell viability loss from 20% (column 3) to 15% (column 4) and 8% (column 5) with 0.5 and 2 μg Nrf2 transfection, respectively (Fig. 5D, $P < 0.05$), with a significant recover in cell viability loss ($P > 0.05$). Both types of cell damage decrease in a Nrf2 concentration- dependent manner.

<Figure 5>

DISCUSSION

Refractoriness of HO-1 protein following the second dose of UVA irradiation

Refractoriness of HO-1 gene activation to a second treatment with hemin was first observed in chicken embryo liver cells (8). Later it was shown that, following a second treatment with a moderate, physiological dose of UVA irradiation or hemin, human skin fibroblasts FEK4 had acquired refractoriness to activate the HO-1 gene as monitored at the level of transcription (9). In this study we extended these observations to the protein level by studying the development of refractoriness to re-induction of HO-1 protein following a second treatment with UVA radiation at different time intervals. As expected, refractoriness to HO-1 protein induction develops at later times than refractoriness to HO-1 mRNA accumulation and the former occurs maximally with a 48 h interval time. The initial UVA inducedHO-1 mRNA accumulation is back to normal at 16 h following UVA irradiation (9). The maximal reduction in HO-1 protein levels (refractoriness) occurred following the second dose of UVA irradiation when cells were incubated for 72 h between two doses of UVA irradiation. The apparent lack of refractoriness to HO-1 induction for UVA/UVA treatment at a 24 h interval may be due to increased amount of HO-1 protein as observed in the relevant controls (UVA/-): still remaining high level 24 h after irradiation, i.e. the pre-irradiated cells still had 3-4 times higher level of HO-1 protein as compared to the sham control. It is notable that an interval time of 24 h was required to obtain a maximal reduction in re-induction of HO-1 activity by a moderate dose of UVA irradiation in a mouse model (18) indicating either a species, or *in vitro* versus *in vivo* difference.

Both UVA and heme treatment can cause refractoriness to induce HO-1 protein by a second treatment. Cadmium chloride (cadmium) increased both HO-1 and Nrf2 levels (19) but it does not lead to the refractoriness to any type of second treatment. This is similar to previous studies with sodium arsenite, another strong HO-1 inducer and it is likely that this is because these compounds do not alter heme levels (1,9,15,20). A combination of heme and UVA radiation treatments was not given because heme

sensitises cells against UVA mediated cell death (9,16). The UVA induction of HO-1 level is correlated to the extent of UVA released microsomal heme, and reduced heme levels were found 48 h following UVA irradiation (16). Refractoriness observed following a second treatment with UVA radiation or heme might link to the reduction of heme levels because it is known that the first treatment strongly increases HO-1 levels(9). This result was supported by our previous study showing that the inhibition of heme synthesis significantly reduced UVA-induced HO-1 protein levels (12,14).

Refractoriness of HO-1 protein may due to Nrf2 refractoriness

Nrf2 is involved in dissipating the stress and providing a protective response following UVA induction of oxidized phospholipids in skin cells (21). Loss of Nrf2 increases UVA-mediated apoptosis in mouse skin fibroblasts (22). The protein can be activated as a result of stabilisation, translocation and nuclear accumulation (6). Our results show that Nrf2 silencing leads to an enhanced refractoriness to HO-1 induction after a second treatment with both UVA radiation and heme; and that an increase in Nrf2 protein leads to a reduced refractoriness to HO-1 induction after a second treatment with UVA radiation. These results indicate that pre-irradiation not only leads to reduced total levels of Nrf2 protein accumulation following a second dose, but it may also result in reduced Nrf2 nuclear accumulation. UVA treatment leads to both a reduced level of Nrf2 activation, and increased Bach1 activation when a second dose of UVA is applied. Reduction of Nrf2 leads to a lower induction of HO-1 by UVA irradiation (12) while an increase of Nrf2 will lead to high induction of HO-1 by UVA irradiation. Conversely, Bach1 reduction leads to increased levels of HO-1 after UVA irradiation in these cells (15). The alteration of Nrf2 up-regulation by either heme or UVA irradiation may therefore be involved in the HO-1 refractoriness response.

Both Nrf2 and Bach1 translocation and expression are modulated by UVA radiation which therefore modulates UVA-induced HO-1 expression (12-15). We may expect that lack of free heme following an increased activity of HO-1 will prevent stabilisation of Nrf2. The consequent reduction in Nrf2, together with the stabilisation

and increase in Bach1 levels will both contribute to diminish HO-1 re-induction by a second UVA dose.

Repeated UVA irradiations cause more cell damage and Nrf2 is implicated in protection from a second treatment with UVA irradiation

Loss of Nrf2 sensitises cells to UVA radiation induced damage (12,22). Nrf2 is involved in protecting many different cell lines against oxidative damage since it up-regulates detoxifying phase II enzymes, especially HO-1. The protective effect of Nrf2 against UVA radiation as well as several other damaging agents has been demonstrated in skin keratinocytes and fibroblasts (12,13,22). A previous study indicated that a second regime of UVA radiation causes the same extent of free iron release as the first dose and thus may cause similar or increased cell membrane damage in FEK4 cells (17). These findings are in agreement with studies by Merwald (23), who found that the fractioned UVA exposure precedes a greater rate of cells mortality compare with single regime. However, they are in contrast to a previous finding from Tyrrell's laboratory that fully confluent FEK4 cells are protected by pre-irradiation with an optimum dose of UVA radiation (250 kJ/m^2) from high doses of UVA radiation (750 kJ/m^2) (24). In the previous study, protection was found 24 h following UVA treatment, when HO-1 protein levels are significantly higher (9,24) (Fig. 1). Furthermore, fully confluent cells are generally more resistant to UVA irradiation than less confluent cells.

A related study in a mouse model reported that UVA radiation induced refractoriness to HO-1 induction by a second UVA treatment with a 24 h time interval and this is linked to reduced immunoprotection (18). This result further indicated that repeated UVA irradiation may cause more damage to skin when compared with a single exposure. The fact that a second exposure with an optimum dose (250 kJ/m^2) of UVA can cause and enhance cell damage relative to a single dose of UVA may be attributable to a lower expression of Nrf2 protein under these conditions. Repetitive UVA damage may be reduced by an increase in Nrf2 levels, which further implies that activation of Nrf2 contributes to the protection of human skin fibroblasts against oxidative damage.

However other molecules may also be involved (12,13,25,26). It has been argued that Nrf2 signalling may offer a protective role in aging, including photoaging and it may play a role during keratinocyte differentiation (27,28). Further, increase in Nrf2 levels by moderate proteasome activation may affect the aging process and the cellular response to oxidative stress in human fibroblasts (29). While the Nrf2/HO-1 system may protect human skin cells against UVA-mediated damage, the activation may increase tumour progression (30).

In summary, we have demonstrated that UVA radiation causes refractoriness of human skin fibroblasts to re-induction of both HO-1 and Nrf2 by a second dose of UVA irradiation and that both Nrf2 and Bach1 might cooperate in HO-1 refractoriness. While our data support the concept that Nrf2 may have a protective function in skin fibroblasts upon single and multiple UVA treatments, the involvement of Nrf2 in human skin protection *in vivo* remains to be determined. Repeated introduction of human skin cells to moderate and high doses of UVA irradiation results in enhanced cell damage and Nrf2 may offer protection under such conditions.

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523

FIGURE CAPTIONS

Figure 1. Dose and time dependence of induction of HO-1 protein by UVA in the human skin fibroblastFEK4. Cell lysates were harvested at 8 h after UVA radiation (A), or at the indicated times following exposure to 250 kJ/m2UVA radiation (B). In each lane, total protein (40 μg) was separated on 12% SDS-PAGE gels, transferred to PVDF membranes and probed for HO-1 and actin. Quantification of optical densities of individual bands was carried out using digital densitometry. Values were normalised with respect to the actin signals. The expression levels of HO-1 are shown as a fold induction relative to the sham irradiated control (set to 1) on the bar graph. Data are presented as mean ± S.E (n = 4). *, P<0.05， **, P<0.01 vs. the sham control.

Figure 2. Treatments with UVA irradiation and heme, but not cadmium lead to refractoriness to re-induction of HO-1 protein. Cells were treated with 250 kJ/m^2 UVA irradiation (A), 5 μMheme (B) or 2.5 μM cadmium (C) for 1 h, and then incubated in conditional medium (cMedium) for 24, 48, 72h (Figure 2A,UVA irradiation), and 60 h (Figure 2B and C, heme or cadmium) before a second treatment under similar conditions. The cells were harvested at 12 h after second treatment and assayed for the expression of HO-1 protein. Values were normalised by actin loading, the levels of HO-1, a relative fold induction to the sham irradiated control (set to 1) were shown in the bar graph. Data are presented as mean ± SE (n = 4). *P<0.05**,** **P<0.01 vs. the sham control. Cd: cadmium. 1^o: first treatment, 2^o: second treatment.

Figure 3.The effect of a second UVA radiation treatment on total and nuclear accumulation of Nrf2 and Bach1 protein.FEK4 cells were pre-treated with 250 kJ/m^2 UVA irradiation, and re-incubated in cMedium for 48 h, followed by a second dose of UVA. Cells were collected at the indicated times post irradiation. A. Cells grown on coverslips were collected and fixed, then permeabilised and immunostained with anti-Nrf2 antibody (1:200, green: 3A), Bach1 (1:300, Red: 3B). Hoechst dye (shown in red)

was used to visualise cell nuclei. 3C.Western blotting was performed with 35µg/lane of whole cell protein as described previously; Actin signals were determined for all samples and used to normaliseNrf2 Bach1 and HO-1, protein levels. Relative Nrf2 (3D), Bach1 (3E) and HO-1 (3F), levels in the samples were expressed as fold increases. Data are presented as mean ± SE (n = 4). * P<0.05vs.the relevant single irradiated control (-/UVA).

Figure 4. Effect of modulation of Nrf2 levels on refractoriness of HO-1 to induction by a second treatment with UVA. FEK4 cells were transiently transfected with vehicle control (–), negative scrambled control siRNA (Sb, 30 nM), 5 and 30 nM of siNrf2, using siPORTNeoFX transfection reagent (Ambion) as described in materials and methods (A and B). Cells were transiently transfected with vector control (V), Nrf2 constructs (0.5 and 2 µg), using lipofectamine transfection reagent as described in materials and methods (C).

Cells were cultured for 60 h, then pre-treated with 250 kJ/m^2 UVA irradiation (A) or 5 µM heme (B), and re-incubated in cMedium for an additional 60 h, followed by a second dose of either 250 kJ/m^2 UVA irradiation or heme, and collected 12 h later; Cells were transfected with Nrf2 as described in Fig. 4, then pre-treated with 250 kJ/m^2 UVA irradiation, and re-incubated in cMedium for an additional 48 h, and collected at 6 h following UVA irradiation (C). Western blotting of HO-1 protein and PCR for mRNA was performed as described previously. Relative HO-1 levels were expressed as a fold increase after being normalised by actin protein (A and B), or C for GAPDH mRNA signals. Data are presented as mean ± SE (n = 4). *, P <0.05 vs. the relevant control.

Figure 5. Nrf2 silencing further increased re- UVA irradiation induced cell damage; while Nrf2 overexpression reduced re- UVA irradiation induced cell damage. FEK4 cells were transfected with siNrf2 (A and B) or Nrf2 (C) for 48 h, then

580 either sham or UVA-irradiated and re-incubated, prior to a second UVA treatment (as
581 described in Fig. 4). At 8 h following the re-irradiation, membrane damage (A, C) and
582 cell viability (B, D) were determined by the LDH and MTS assays, respectively. The
583 percentage of LDH leakage and viability were expressed as relative fold changes
584 compared with the sham irradiated control, set as 1 (A) and 100 (B), respectively. Data
585 are presented as mean ± SE (n = 4). * $P<0.05$ vs. the relevant control.